Clinical Cancer Research

The Effect of Preservative and Temperature on the Analysis of Circulating Tumor DNA

Sonya Parpart-Li[1], Bjarne Bartlett[2,3], Maria Popoli[2,3], Vilmos Adleff[2], Laura Tucker[1], Rebecca Steinberg[1], Andrew Georgiadis[1], Jill Phallen[2], Julie Brahmer[2], Nilo Azad[2], Ilene Browner[2], Daniel Laheru[2], Victor E. Velculescu[2], Mark Sausen[1], and Luis A. Diaz Jr[2,3]

Abstract

Purpose: Analysis of genomic alterations in cell-free DNA (cfDNA) is evolving as an approach to detect, monitor, and genotype malignancies. Methods to separate the liquid from the cellular fraction of whole blood for circulating tumor DNA (ctDNA) analyses have been largely unstudied, although these may be a critical consideration for assay performance.

Experimental Design: To evaluate the influence of blood processing on cfDNA and ctDNA quality and yield, we compared the cfDNA levels in serum with those in plasma. Given the limitations of serum for ctDNA analyses, we evaluated the effects of two plasma processing approaches, K_2EDTA and Cell-Free DNA BCT (BCT) tubes, on cfDNA and ctDNA recovery. A total of 45 samples from nine patients with cancer were collected in both tube types. Once collected, blood was processed into plasma immediately or kept at room temperature and processed into plasma at 1, 3, 5, or 7 days.

Results: As early as 24 hours after collection, plasma isolated from blood collected in K_2EDTA tubes contained an elevated level of cfDNA that increased over time compared with BCT tubes where no significant increase in cfDNA levels was observed. When samples from an additional six patients with cancer, collected in the same manner, were stored at 4°C in K_2EDTA tubes over the course of 3 days, total cfDNA and ctDNA levels were comparable between samples collected in BCT tubes. At day 3, there was a trend toward a decrease in ctDNA levels in both tubes that was more pronounced when measuring the mutant allele fraction for cases stored at 4°C in K_2EDTA tubes.

Conclusions: In summary, methods of blood processing have a strong influence on cfDNA and ctDNA levels and should be a consideration when evaluating ctDNA in peripheral circulation. *Clin Cancer Res; 23(10); 2471–7. ©2016 AACR.*

Introduction

The clinical applicability of liquid biopsies is becoming increasingly evident with the emergence of novel targeted therapies (1–3) combined with next-generation technologies for assessment of tumor-specific sequence and structural alterations in cell-free DNA (cfDNA) derived from blood (4–12). These noninvasive approaches provide a method to evaluate tumor-specific alterations present in the tumor sample without the need for a tissue biopsy through direct identification of circulating tumor DNA (ctDNA) similar to noninvasive prenatal screening tests (NIPT) that scan a maternal blood sample for genetic conditions in the fetus. On average, the fetal fraction of DNA in maternal plasma ranges from 10% to 30% (13–15). In contrast, ctDNA is often present at significantly lower levels and can be considerably affected by less robust sample collection techniques as there may be only a few mutant DNA fragments per milliliter (mL) of plasma (6, 16). Therefore, ctDNA levels may depend upon how the cell and cell-free components were isolated into plasma or serum, especially as cfDNA has a very short half-life (6, 17, 18). Once cfDNA is released into circulation from apoptotic or necrotic cells, the half-life is estimated to be about 2 hours (6, 18). Although the rapid turnover of cfDNA allows for sophisticated monitoring of tumor dynamics, blood collection procedures should be carefully considered if the end goal is to detect ctDNA. To date, plasma has been the primary cell-free blood component used for ctDNA analyses, but studies have been conducted using serum (5). To isolate plasma, whole blood is anticoagulated to prevent clotting of the cellular component, and following centrifugation, the cell-free supernatant is removed. When three commonly used anticoagulants [di-potassium ethylenediaminetetraacetic acid (K_2EDTA), lithium heparin, and sodium citrate] were evaluated in their ability to stabilize cfDNA levels after venipuncture, it was found that total plasma DNA concentrations were stable with all 3 anticoagulants if whole blood was processed into plasma within 6 hours. However, K_2EDTA was the anticoagulant with the smallest change in cfDNA levels after 6 hours (19). To better understand the influence of preanalytic whole blood processing on cfDNA levels, we evaluated several conditions to find the optimal approach.

Materials and Methods

Patient selection and sample processing

All biologic specimens utilized in this study were obtained from patients enrolled in a study approved by the Johns Hopkins

[1]Personal Genome Diagnostics, Inc., Baltimore, Maryland. [2]The Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine, Baltimore, Maryland. [3]The Swim Across America Laboratory at Johns Hopkins, Baltimore, Maryland.

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Corresponding Author: Luis A. Diaz Jr, Johns Hopkins, CRB I, Room 590, Baltimore, MD 21231. Phone: 410-955-8878; Fax: 410-955-0548; E-mail: ldiaz1@jhmi.edu

doi: 10.1158/1078-0432.CCR-16-1691

Translational Relevance

Preserving the integrity of ctDNA over an extended period is crucial to achieve the high sensitivity required for downstream molecular analyses. In this study we preanalytically processed blood samples in two tube types, specific for the collection of plasma, under various environmental conditions to determine the best approach for maintaining ctDNA quality and yield. Notably, we found that the plasma fraction of whole blood is more desirable than the serum fraction for the analysis of ctDNA. The most commonly used tube type for plasma collection, K_2EDTA, stored at 4°C maintains ctDNA in a manner similar to Cell-Free DNA Blood Collection tubes (Streck) stored in ambient conditions. At 4°C, cell lysis is prevented, reducing the amount of total gDNA released into the plasma and allowing for variants in ctDNA to be more readily detected. These results will inform the design of studies that rely on collecting plasma for ctDNA analysis.

University Institutional Review Board and provided informed consent. Patients whose tumors had been sequenced as part of standard clinical care and had "hot spot" mutations in *KRAS*, *EGFR*, or *BRAF* were recruited into this study.

Serum was prepared after collection of the whole blood into tubes containing no anticoagulant. Whole blood was allowed to clot by leaving it undisturbed at room temperature for 15 to 30 minutes. The clot was removed by centrifuging at 1,000 to 2,000 × *g* for 10 minutes in a refrigerated centrifuge. The resulting supernatant is designated serum and was carefully aspirated from the tube without disturbing the clot (cells) and transferred in 1-mL aliquots into 1.5-mL Eppendorf tubes and then centrifuged a second time at 18,400 × *g* for 10 minutes to remove cellular debris. Serum aliquots were then transferred to a new 1.5-mL Eppendorf tube and frozen at −80°C.

Plasma was prepared from whole blood collected in K_2EDTA or BCT tubes. Cells were removed from the plasma by centrifugation at 375 × *g* for 10 minutes at room temperature. The resulting supernatant is designated plasma and was carefully aspirated from the tube without disturbing the buffy coat layer (cells) and transferred in 1-mL aliquots into 1.5-mL Eppendorf tubes and then centrifuged a second time at 18,400 × *g* for 10 minutes to remove cellular debris. Plasma aliquots were then transferred to a new 1.5-mL Eppendorf tube and frozen at −80°C.

Plasma processing at room temperature

Ten tubes of peripheral blood (~10 mL per tube) were collected from each patient with tumors that harbored *KRAS* mutations. Of these 10 tubes, 5 were K_2EDTA tubes and the 5 tubes were BCT tubes. After venipuncture, the tubes were inverted 10 times immediately after the blood draw. Whole blood was kept at room temperature and processed into plasma at time points (0, 24, 72, 120, 168 hours), except for the baseline sample (time 0) that was processed into plasma (as described above) within 4 hours of venipuncture.

Plasma processing at room temperature and 4°C

When the influence of temperature on ctDNA levels was assessed, 12 tubes of peripheral blood (~10 mL per tube) were collected from 6 patients with tumors that harbored *KRAS* mutations. Of these 12 tubes, 6 were K_2EDTA tubes and the 6 tubes were BCT tubes. After venipuncture, the tubes were inverted several times. K_2EDTA tubes and BCT tubes were equally split into two sets: one set was kept at 4°C whereas the other set was stored at room temperature and processed into plasma at time points 0, 24, and 72 hours, except for the baseline sample (time 0) that was processed into plasma (as described above) within 4 hours of venipuncture.

cfDNA extraction and mutant analysis

Circulating cfDNA was extracted from plasma using the QIAamp Circulating Nucleic Acid Kit (Qiagen, Catalog #55114). The purified DNA concentration was determined using Qubit dsDNA HS Assay Kit (Life Technologies, Catalog #Q32854). All extracted DNA was analyzed with the appropriate droplet digital PrimePCR assay (BioRad, Catalog #100-31246 and #100-31249) on the BioRad QX200 droplet digital PCR system to determine the number of wild-type and mutant genomic equivalents. The mutant fractional abundance was calculated as a percentage of the total DNA present at each time point. The fragment size of cfDNA derived from plasma and serum was measured using the High Sensitivity DNA Kit on the 2100 Bioanalyzer (Agilent, Catalog #5067-4627).

Circulating cfDNA from healthy controls was extracted and quantified as described above. Genomic libraries were created from the total yield of extracted DNA using NEB enzyme mixes for End Repair (cat# E6050), A-tail (cat# E6053), and ligation (cat# E6056, incorporating 8 pooled adapter oligonucleotides generated by IDT) followed by 12 cycles of PCR and purified as described previously using Agencourt AMPure XP beads (Beckman Coulter; ref. 20). A 58-gene panel (Supplementary Table S4) interrogating 80,930 nucleotides was employed to detect sequence mutations by next-generation sequencing, using the Agilent SureSelect in-solution targeted enrichment system (Agilent; ref. 21).

Targeted regions were captured in-solution using a custom SureSelect kit according to the manufacturer's instructions. The captured DNA library was immobilized from the beads using 200 μL of a 95% Formamide and 10 mmol/L EDTA solution at 65°C for 5 minutes. Once separated from the beads, the DNA was purified using Agencourt AMPure XP beads (Beckman Coulter) in a ratio of 1.0× to 1.8× ratio of captured DNA library to beads and washed using 70% ethanol according to manufacturer's instructions. The captured DNA library was amplified and purified as described previously (20). The captured genomic library underwent 100-bp paired-end sequencing on a HiSeq 2500 instrument (Illumina) to obtain an average of 26,574× total coverage in the targeted region of interest (Supplementary Table S5), with variant identification performed as previously described (21, 22).

Statistical analyses

The statistical difference between BCT and K_2EDTA tubes at various time points as well as the difference in total genomic equivalents or mutant allele fraction (MAF) between serum and plasma was calculated using a paired, 2-tailed Student *t* test where $P < 0.05$ was considered significant (GraphPad Prism v5.00). To study sample size, a power analysis was performed in R (v3.2.4). Six samples were determined to provide an 80% power for the detection of a 10% difference between blood collection tube types ($P < 0.05$, SD = 7). One sample set was removed from the analysis

as the baseline MAF was <1% and had the potential to confound these findings. For a reduced study size of 5, the study was determined to provide 67% power for the detection of a 10% difference between blood collection tube types.

Results

Total and tumor-derived cfDNA levels in plasma and serum

To evaluate the yield of cfDNA between serum and plasma, the quantity of total genome equivalents and the MAF were compared between serum and plasma collected from patients with stage II and III pancreatic cancer ($n = 46$ and $n = 51$, respectively). All cases were known to harbor mutations in *KRAS* at p.G12C, p.G12D, p.G12I, p.G12R, p.G12S, p.G12V, or p.G13D positions. DNA extracted from serum was analyzed for mutations known to be present in the matched tumor using standard droplet digital PCR assays. We compared our results in serum with a similar dataset we had previously analyzed for mutations in plasma from 51 stage-matched patients with pancreatic cancer from a study by Sausen and colleagues (20). Total genome equivalents were significantly greater in the DNA obtained from serum specimens ($P < 0.001$, Fig. 1A), whereas the observed MAF was significantly higher in the DNA obtained from plasma than from serum specimens ($P < 0.001$, Fig. 1B). While distinct peaks of cfDNA were observed in plasma with the largest fraction measuring 155 base pairs on average, the cfDNA fragments in the serum varied considerably in size ranging from 150 to 2,000 base pairs (Fig. 1C).

The effect of immediate and delayed plasma preparation on total and tumor-derived cfDNA levels

Whole blood from 9 patients with colorectal cancer, where the tumor genotype was known to harbor a mutation in *KRAS*, *EGFR*, or *BRAF*, was collected and monitored to assess the stability of DNA obtained from K_2EDTA tubes and BCT tubes at room

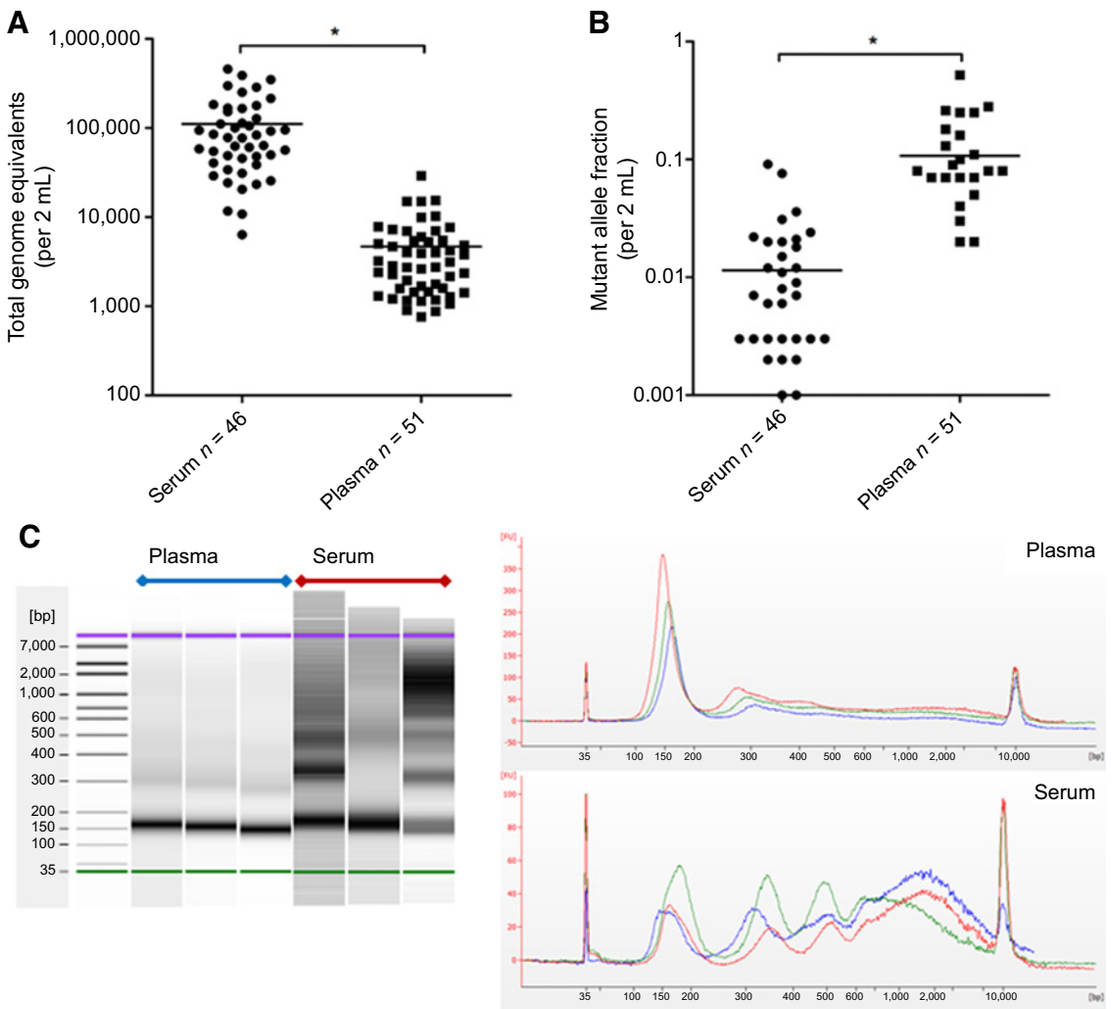

Figure 1.

Levels of gDNA in serum and plasma of patients with pancreatic cancer. **A,** Average total genomic equivalents were significantly lower in plasma than in serum (*, $P < 0.001$, Student *t* test). **B,** Observed MAF was significantly lower in serum than in plasma (*, $P < 0.001$, Student *t* test). **C,** cfDNA was observed at one major peak between 150 and 200 base pairs and one minor peak around 300 base pairs in plasma. In serum, cfDNA was observed at multiple peaks between 150 and 2,000 base pairs.

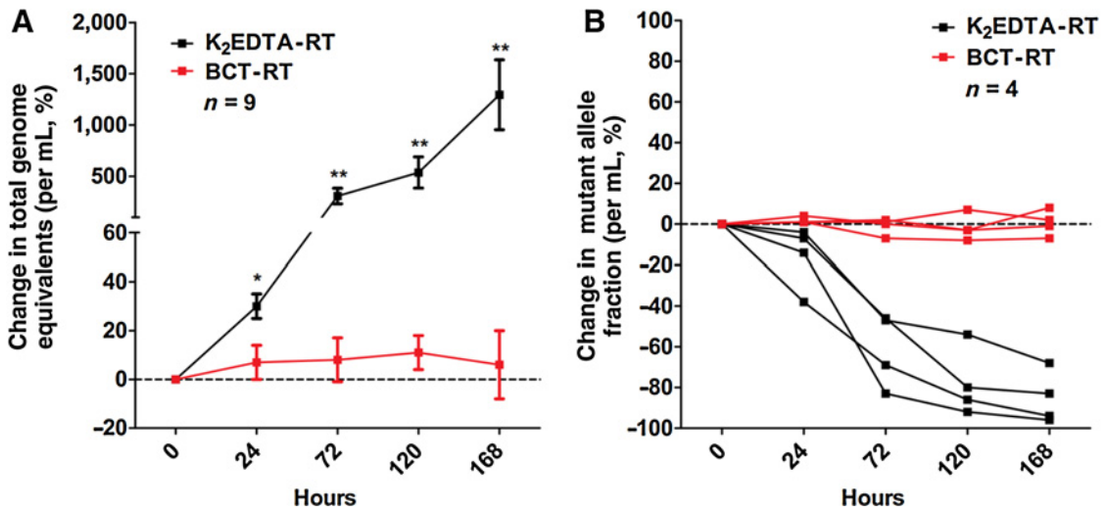

Figure 2.

Characteristics of cfDNA obtained from plasma isolated from blood stored at room temperature. **A,** Percentage change in total genome equivalents significantly increased in K_2EDTA tubes compared with BCT tubes at room temperature ($P < 0.05$ at 24 hours; $P < 0.01$ at 72, 120, and 168 hours; Student t test). The percentage change in total genome equivalents significantly increased within the K_2EDTA tube type ($P < 0.005$ between 0 and 168 hours, Student t test) but did not change over time within the BCT tubes ($P > 0.05$ for all time points, Student t test). *, Significant difference between K_2EDTA and BCT tubes. **B,** Observed MAF was stable over time when blood was preserved in BCT tubes but decreased when blood was preserved in K_2EDTA tubes (representative 4 cases shown). *, $P < 0.05$; **, $P < 0.01$.

temperature over a period of 7 days (Supplementary Fig. S1 and Supplementary Table S1). During this time, the change in overall genome equivalents observed in DNA obtained from BCT tubes remained stable compared with K_2EDTA tubes stored at room temperature ($P < 0.005$ between 0 and 168 hours, Fig. 2A, Supplementary Table S2). Within 24 hours, a significant increase in the number of total genome equivalents was observed in the DNA obtained from K_2EDTA tubes, which continued to increase over time. After 1 week, there was a 13-fold increase in total genome equivalents per milliliter in K_2EDTA tubes, whereas the percentage increase of total genome equivalents per milliliter obtained from BCT tubes was 6%. For cases where the MAF was >1% in both tube types, the observed MAF was stable in blood collected and stored in BCT tubes but decreased considerably in blood collected and stored in K_2EDTA tubes (Fig. 2B, one time point in P1 was unable to be evaluated and therefore excluded from analysis, Supplementary Table S2).

The effect of temperature on total and tumor-derived cfDNA levels

To determine whether storage of K_2EDTA tubes at 4°C could increase the allowable time interval between blood collection and fractionation, blood was collected in both K_2EDTA and BCT tubes from 6 *KRAS*- or *EGFR* mutant–positive patients with cancer (Supplementary Fig. S1 and Supplementary Table S1) and held at either room temperature (Streck BCT) or at 4°C (K_2EDTA) for up to 72 hours. When K_2EDTA tubes were stored at 4°C, there was no significant increase in yield of total genomic equivalents over the 3-day period (Fig. 3A, Supplementary Table S3) in contrast to the observed increase in total genomic equivalents when whole blood was kept at room temperature and stored in K_2EDTA tubes. We also analyzed the change in total mutant DNA fragments per milliliter and the change in MAF over the same 3-day period. The percentage change in the number of mutant DNA fragments initially increased at 24 hours in both tubes but decreased by 4% (Streck BCT) or 21% (K_2EDTA) at 72 hours, with no significant difference observed between K_2EDTA and BCT tubes ($P > 0.05$ at each time point, Fig. 3B, Supplementary Table S3). The MAF decreased in plasma isolated from K_2EDTA tubes and BCT at 24 and 72 hours with a statistically significant difference observed between tube types ($P < 0.05$, Fig. 3C, Supplementary Table S3) at 72 hours. There was an observable difference in gDNA levels between plasma DNA extracted at baseline (within 4 hours of blood draw) and plasma DNA extracted at 72 hours in K_2EDTA tubes stored at 4°C or room temperature (Fig. 3D), whereas BCT tubes show minimal differences in total cfDNA levels regardless of storage temperature or time (Fig. 3E). In all cases, the trends in cfDNA and ctDNA levels were consistent between K_2EDTA tubes and BCT in each series tested.

The effect of immediate and delayed plasma preparation on plasma volume

In addition to total genomic equivalents, we evaluated the volume of plasma obtained from fractionated blood at room temperature over the 7-day period. We found that the volume of plasma obtained decreased in both tube types over time using the same fractionation conditions (Fig. 4A). By day 7 (168 hours), less than 2 mL of plasma was fractionated from each 10 mL Vacutainer tube (Streck or K_2EDTA) containing 8 to 10 mL of whole blood.

Next-generation sequencing quality and specificity

The number of mutations between BCT and K_2EDTA tubes was analyzed using a hybrid-capture, next-generation sequencing approach. Three BCT and 3 K_2EDTA tubes of whole blood (~10 mL per tube) from 3 healthy individuals were analyzed for sequence mutations over a 72-hour period where BCT tubes were

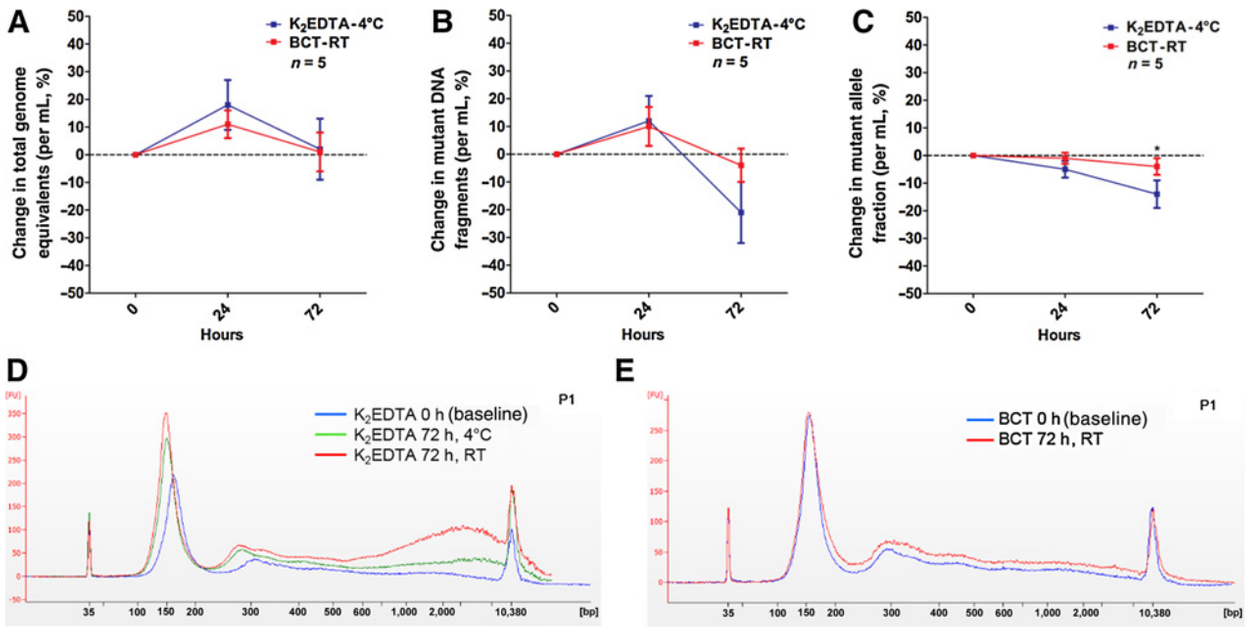

Figure 3.

The effects of blood tube collection storage conditions on cfDNA and ctDNA yield. **A,** Percentage change in total genome equivalents per milliliter remained stable using both tube types up to 3 days when K_2EDTA tubes were stored at 4°C and BCT tubes were stored at room temperature. There was no significant difference between or within the tube types at any time point ($P > 0.05$, Student t test). **B,** Percentage change in mutant DNA fragments decreased at 72 hours in both BCT (−4%) and K_2EDTA (−21%) tubes; however, the decrease was not significant within or between tube types at any time point ($P > 0.05$, Student t test). **C,** Percent change in MAF decreased after 24 hours and continued to decrease at 72 hours in both BCT (−1%, −4%) and K_2EDTA (−5%, −14%) tubes, respectively. The difference observed between K_2EDTA and BCT tubes was significant at 72 hours ($P < 0.05$, Student t test). There was no significant difference observed within either tube type at any time point ($P > 0.05$, Student t test). **D,** Bioanalyzer trace shows an observable increase in gDNA (>1 kbp) in K_2EDTA tubes after 72 hours. **E,** Total gDNA levels in BCT tubes at 72 hours compared with the baseline reading while stored at room temperature (RT). *, $P < 0.05$.

stored at RT while K_2EDTA tubes at 4°C (Supplementary Table S5). Using a targeted 58-gene panel designed to cover 80,930 nucleotides and detect sequence mutations in plasma, the observed per base error rate, calculated as the number of bases mutated divided by the number of targeted bases, was similar between plasma isolated from K_2EDTA and BCT tubes (0.00041% vs. 0.00014%, $P = 0.28$, Student t test). Consistent with previous findings, the yield of cfDNA in healthy individuals was significantly lower than the yield in diseased individuals ($P < 0.02$, Student t test, Supplementary Table S6; refs. 4, 23, 24).

Discussion

Methods of whole blood processing, preservation, and storage are important considerations for the analysis of ctDNA. A common question is whether serum or plasma are equivalent for cfDNA analyses. It is clear that the abundance and nature of the cfDNA is very different in these 2 fractions of whole blood. Serum contains high-molecular-weight DNA, likely attributed to how serum is produced by clotting whole blood into a solid and liquid phase. This relatively violent process results in the release of cellular DNA into the cell-free liquid component by lysis and apoptosis (Fig. 1). In contrast, plasma is produced using an anticoagulant (potassium EDTA, sodium citrate, lithium heparin) and the cellular (solid) component gently separates from the plasma (liquid) component resulting in cfDNA that is uniform in size (median size of 180 bp). This is likely the reason that K_2EDTA tubes are recommended by the International Council for Standardization in Hematology (ICSH) and the Clinical and Laboratory Standards Institute (CLSI) as the ideal tube for molecular testing (such as routine PCR-based molecular diagnostics and HIV viral load testing) because the anticoagulant dipotassium salts preserve cellular components (25, 26) and prevent contamination and dilution with cellular DNA that may interfere with analyses of cfDNA. In addition, EDTA salts have been shown to inhibit DNAse activity and thereby help preserve cfDNA levels *ex vivo* during storage, transport, and processing (27). Di- and tri-potassium (K2 and K3) salts of EDTA are standard anticoagulants that have been used in studies with cfDNA. K_2EDTA is spray-dried on the walls of the tube rather than added in liquid form, as is the case for K_3EDTA. The spray-dried coating has been shown to prevent both cell shrinkage and other osmotic effects on blood cells more effectively than in liquid form (28, 29). Several studies have evaluated cfDNA and ctDNA levels when whole blood was stored and preserved with K_3EDTA. These analyses show varying results, many with findings similar to ours when evaluating cfDNA and ctDNA levels in stored whole blood (27, 28, 30–33).

In our study, we compared K_2EDTA tubes with BCT tubes, which are intended to preserve cfDNA through proprietary components that are free of formaldehyde. cfDNA levels remained stable in BCT tubes over a 7-day period even when whole blood was stored at room temperature and likewise cfDNA levels were stable when whole blood was stored at 4°C in K_2EDTA tubes for 24 hours. Not surprisingly, temperature has been shown to have a strong influence on peripheral blood storage in K_2EDTA-coated tubes. Prior studies have shown that storage of whole blood in

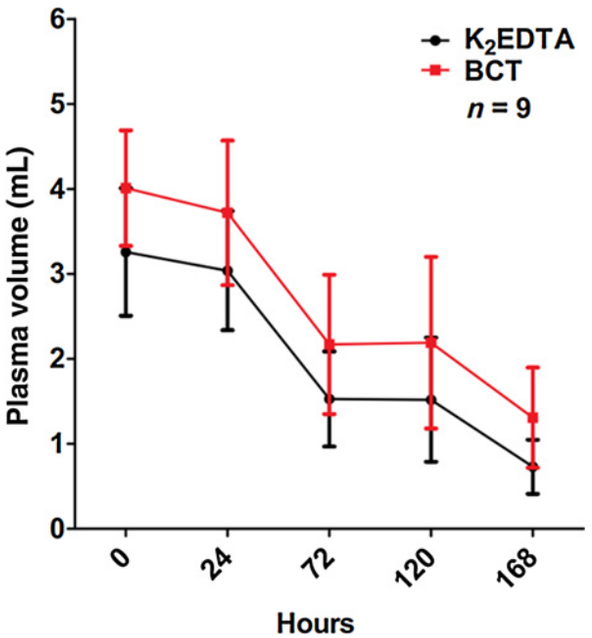

Figure 4.
The effects of blood tube collection storage conditions on the volume of plasma collected. Volume of plasma collected from each tube type decreased as the tube remained at room temperature over a 1-week period. There was no significant difference between the plasma volumes collected in K_2EDTA versus BCT tubes over time (Student *t* test).

K_2EDTA tubes at 4°C results in the stability of leukocytes and plasma proteins for an extended period (29). Likewise, storage of whole blood at 4°C preserves both total cfDNA and ctDNA at levels equivalent to the BCT tubes. In contrast, when blood was stored at room temperature in K_2EDTA-coated tubes, there was a massive release of cfDNA presumably by cells undergoing unorganized death by lysis or necrosis as demonstrated by the high-molecular-weight DNA fragments found in the plasma after 24 hours (Figs. 2A and 3D). At room temperature, there was also a concomitant decrease in the fraction of ctDNA (Fig. 2B) likely from the dilution of the mutant DNA fragments by wild-type DNA released by the lysed lymphocytes. We also noted that at room temperature, the volume of plasma recovered significantly decreased regardless of storage tube used and should be considered when storage times exceed 1 day, especially when samples are idle. Furthermore, it appears that using either of these preservatives have no observable effects on the quality of next-generation sequencing analyses of cfDNA.

As the number of ctDNA analyses increase for the determination of mutation profiles in patients with cancer for research and clinical care, the methods used for whole blood preservation and storage before processing into plasma and DNA extraction will influence the quality of analysis. It is clear that minimizing cellular lysis during whole blood storage is of high importance and this can be accomplished using BCT tubes at room temperature or in EDTA tubes stored at 4°C.

Disclosure of Potential Conflicts of Interest

V. Adleff is a consultant/advisory board member for Personal Genome Diagnostics. V.E. Velculescu holds ownership interest (including patents) in and is a consultant/advisory board member for Personal Genome Diagnostics. L.A. Diaz Jr is an employee of Personal Genome Diagnostics; reports receiving speakers' bureau honoraria from Merck; and holds ownership interest (including patents) in PapGene and Personal Genome Diagnostics. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: S. Parpart-Li, D. Laheru, V.E. Velculescu, M. Sausen, L.A. Diaz Jr
Development of methodology: S. Parpart-Li, B. Bartlett, V. Adleff, V.E. Velculescu, M. Sausen, L.A. Diaz Jr
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): B. Bartlett, V. Adleff, L. Tucker, J. Phallen, J.R. Brahmer, N. Azad, I. Browner, L.A. Diaz Jr
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Parpart-Li, R. Steinberg, A. Georgiadis, J.R. Brahmer, V.E. Velculescu, M. Sausen, L.A. Diaz Jr
Writing, review, and/or revision of the manuscript: S. Parpart-Li, B. Bartlett, V. Adleff, A. Georgiadis, J.R. Brahmer, I. Browner, D. Laheru, V.E. Velculescu, M. Sausen, L.A. Diaz Jr
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): B. Bartlett, M. Popoli, A. Georgiadis, L.A. Diaz Jr
Study supervision: V.E. Velculescu, M. Sausen, L.A. Diaz Jr

Acknowledgments

We would like to thank S. Bonerigo and A. Ryan (Johns Hopkins) for their clinical coordination.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received July 6, 2016; revised October 3, 2016; accepted October 23, 2016; published OnlineFirst November 8, 2016.

References

1. Alix-Panabieres C, Pantel K. Clinical applications of circulating tumor cells and circulating tumor DNA as liquid biopsy. Cancer Discov 2016;6:479–91.
2. Diaz LA Jr, Bardelli A. Liquid biopsies: genotyping circulating tumor DNA. J Clin Oncol 2014;32:579–86.
3. Heitzer E, Ulz P, Geigl JB. Circulating tumor DNA as a liquid biopsy for cancer. Clin Chem 2015;61:112–23.
4. Dawson SJ, Tsui DW, Murtaza M, Biggs H, Rueda OM, Chin SF, et al. Analysis of circulating tumor DNA to monitor metastatic breast cancer. N Engl J Med 2013;368:1199–209.
5. Diaz LA Jr, Williams RT, Wu J, Kinde I, Hecht JR, Berlin J, et al. The molecular evolution of acquired resistance to targeted EGFR blockade in colorectal cancers. Nature 2012;486:537–40.
6. Diehl F, Schmidt K, Choti MA, Romans K, Goodman S, Li M, et al. Circulating mutant DNA to assess tumor dynamics. Nat Med 2008;14:985–90.
7. Leary RJ, Kinde I, Diehl F, Schmidt K, Clouser C, Duncan C, et al. Development of personalized tumor biomarkers using massively parallel sequencing. Sci Transl Med 2010;2:20ra14.
8. Leary RJ, Sausen M, Kinde I, Papadopoulos N, Carpten JD, Craig D, et al. Detection of chromosomal alterations in the circulation of cancer patients with whole-genome sequencing. Sci Transl Med 2012;4:162ra54.
9. Newman AM, Bratman SV, To J, Wynne JF, Eclov NC, Modlin LA, et al. An ultrasensitive method for quantitating circulating tumor DNA with broad patient coverage. Nat Med 2014;20:548–54.

10. Diaz LA Jr, Sausen M, Fisher GA, Velculescu VE. Insights into therapeutic resistance from whole-genome analyses of circulating tumor DNA. Oncotarget 2013;4:1856–7.
11. Haber DA, Velculescu VE. Blood-based analyses of cancer: circulating tumor cells and circulating tumor DNA. Cancer Discov 2014;4:650–61.
12. Bettegowda C, Sausen M, Leary RJ, Kinde I, Wang Y, Agrawal N, et al. Detection of circulating tumor DNA in early- and late-stage human malignancies. Sci Transl Med 2014;6:224ra24.
13. Canick JA, Palomaki GE, Kloza EM, Lambert-Messerlian GM, Haddow JE. The impact of maternal plasma DNA fetal fraction on next generation sequencing tests for common fetal aneuploidies. Prenat Diagn 2013;33: 667–74.
14. Wang E, Batey A, Struble C, Musci T, Song K, Oliphant A. Gestational age and maternal weight effects on fetal cell-free DNA in maternal plasma. Prenat Diagn 2013;33:662–6.
15. Ashoor G, Poon L, Syngelaki A, Mosimann B, Nicolaides KH. Fetal fraction in maternal plasma cell-free DNA at 11–13 weeks' gestation: effect of maternal and fetal factors. Fetal Diagn Ther 2012;31:237–43.
16. Diehl F, Li M, Dressman D, He Y, Shen D, Szabo S, et al. Detection and quantification of mutations in the plasma of patients with colorectal tumors. Proc Natl Acad Sci U S A 2005;102:16368–73.
17. Fleischhacker M, Schmidt B. Circulating nucleic acids (CNAs) and cancer–a survey. Biochim Biophys Acta 2007;1775:181–232.
18. Lo YM, Zhang J, Leung TN, Lau TK, Chang AM, Hjelm NM. Rapid clearance of fetal DNA from maternal plasma. Am J Hum Genet 1999; 64:218–24.
19. Lam NY, Rainer TH, Chiu RW, Lo YM. EDTA is a better anticoagulant than heparin or citrate for delayed blood processing for plasma DNA analysis. Clin Chem 2004;50:256–7.
20. Sausen M, Phallen J, Adleff V, Jones S, Leary RJ, Barrett MT, et al. Clinical implications of genomic alterations in the tumour and circulation of pancreatic cancer patients. Nat Commun 2015;6:7686.
21. Parpart-Li S, Angiuoli SV, Chesnick B, Galens KG, Jones S, Kadan M, et al. A method for comprehensive genomic analysis of cell free DNA [abstract]. In: Proceedings of the 38th Annual CTRC-AACR San Antonio Breast Cancer Symposium; 2015 Dec 8–12; San Antonio, TX. Philadelphia (PA): AACR; 2015. p. 2405. Abstract nr P2-01-04.
22. Jones S, Anagnostou V, Lytle K, Parpart-Li S, Nesselbush M, Riley DR, et al. Personalized genomic analyses for cancer mutation discovery and interpretation. Sci Transl Med 2015;7:283ra53.
23. Schwarzenbach H, Stoehlmacher J, Pantel K, Goekkurt E. Detection and monitoring of cell-free DNA in blood of patients with colorectal cancer. Ann N Y Acad Sci 2008;1137:190–6.
24. Chun FK, Muller I, Lange I, Friedrich MG, Erbersdobler A, Karakiewicz PI, et al. Circulating tumour-associated plasma DNA represents an independent and informative predictor of prostate cancer. BJU Int 2006;98:544–8.
25. Recommendations of the International Council for Standardization in Haematology for Ethylenediaminetetraacetic Acid Anticoagulation of Blood for Blood Cell Counting and Sizing. International council for standardization in haematology: expert panel on cytometry. Am J Clin Pathol 1993;100:371–2.
26. Adcock DM, Hoefner DM, Kottke-Marchant K, Marlar RA, Szamosi DI, Warunek DJ. Collection, transport, and processing of blood specimens for testing plasma-based coagulation assays and molecular hemostasis assays; Approved Guideline. 5th ed. CLSI Document H21-A5. Wayne, PA: Clinical and Laboratory Standards Institute; 2008.
27. Barra GB, Santa Rita TH, de Almeida Vasques J, Chianca CF, Nery LF, Santana Soares Costa S. EDTA-mediated inhibition of DNases protects circulating cell-free DNA from ex vivo degradation in blood samples. Clin Biochem 2015;48:976–81.
28. Kang Q, Henry NL, Paoletti C, Jiang H, Vats P, Chinnaiyan AM, et al. Comparative analysis of circulating tumor DNA stability In K3EDTA, Streck, and CellSave blood collection tubes. Clin Biochem 2016;49: 1354–60.
29. Banfi G, Salvagno GL, Lippi G. The role of ethylenediamine tetraacetic acid (EDTA) as *in vitro* anticoagulant for diagnostic purposes. Clin Chem Lab Med 2007;45:565–76.
30. Norton SE, Lechner JM, Williams T, Fernando MR. A stabilizing reagent prevents cell-free DNA contamination by cellular DNA in plasma during blood sample storage and shipping as determined by digital PCR. Clin Biochem 2013;46:1561–5.
31. El Messaoudi S, Rolet F, Mouliere F, Thierry AR. Circulating cell free DNA: preanalytical considerations. Clin Chim Acta 2013;424:222–30.
32. Denis MG, Knol AC, Theoleyre S, Vallee A, Dreno B. Efficient detection of BRAF mutation in plasma of patients after long-term storage of blood in cell-free DNA blood collection tubes. Clin Chem 2015;61:886–8.
33. Toro PV, Erlanger B, Beaver JA, Cochran RL, VanDenBerg DA, Yakim E, et al. Comparison of cell stabilizing blood collection tubes for circulating plasma tumor DNA. Clin Biochem 2015;48:993–8.